# SUGAR CHAIN-ADDED HEPARIN-BINDING PROTEIN, METHOD FOR PRODUCING THE SAME AND PHARMACEUTICAL COMPOSITION CONTAINING THE SAME

## DESCRIPTION

### BACKGROUND OF THE INVENTION

The present invention relates to a heparin-binding protein functionalized by covalently bonding thereto a sugar chain, a method for producing the protein and a pharmaceutical composition containing the protein.

It has been known that heparin-binding proteins, among all, those proteins classified into the fibroblast growth factor (hereinafter, referred to as "FGF") family and fibroblast growth factor homologous factors strongly bind to heparin and heparan sulfate (sulfated polysaccharides) by a non-covalent bond. It has been also known that when a heparin-binding protein such as fibroblast growth factor is mixed with a sulfated polysaccharide such as heparin, the biological activity and physical properties of the heparin-binding protein are altered to change its function; sometimes, such a heparin-binding protein may acquire higher function. However, even if a sulfated polysaccharide was mixed with, the expected functionalization of the protein has been limited. Besides, when such a mixture is used as a pharmaceutical composition, unfavorable physiological activity attributable to a free sulfated polysaccharide has caused a problem. To date, there has been reported no protein in which a heparin-binding protein is joined with a sulfated polysaccharide by a covalent bond for the purpose of functionalization of the heparin-binding protein.

In addition, it has never been known to date that artificial

addition of an asparagine-linked sugar chain (hereinafter, referred to as an "N-linked sugar chain") or serine/threonine-linked sugar chain (hereinafter, referred to as an "O-linked sugar chain") to a heparin-binding protein, particularly a protein of the FGF family or a fibroblast growth factor homologous factor, by a covalent bond can functionalize the protein. Furthermore, the general effect which an N-linked sugar chain or O-linked sugar chain could give has not been known. Exceptionally, with respect to FGF-6, the role of the N-linked sugar chain it naturally has was suggested in an *in vitro* translation system, but has not been proved directly. To date, there has been reported no example of joining a heparin-binding protein with an N-linked or O-linked sugar chain by a covalent bond for the purpose of functionalizing the heparin-binding protein.

It is an object of the present invention to improve the function of heparin-binding proteins. It is another object of the invention to establish a heparin-binding protein to which a sugar chain is covalently bonded and a method for producing the protein. It is still another object of the invention to provide a pharmaceutical composition containing the above protein.

## SUMMARY OF THE INVENTION

The present inventors have made intensive and extensive researches toward the solution of the above problems. As a result, the inventors have noted the fact that a sulfated polysaccharide, a glycosaminoglycan, an N-linked sugar chain and an O-linked sugar chain are individually synthesized in living animal bodies as a sugar chain of a glycoprotein. Then, the inventors have found that it is possible to produce a heparin-binding protein having in its molecule a

sulfated polysaccharide, a glycosaminoglycan, an N-linked sugar chain or an O-linked sugar chain covalently bonded thereto by ensuring that a cDNA coding for a peptide to which any of the above sugar chains can be added is ligated to a cDNA coding for the heparin-binding protein, and by then allowing an animal cell to produce the gene product of the ligated cDNA. Furthermore, the inventors have confirmed that the function of the resultant sugar chain-added heparin-binding protein is improved. Thus, the present invention has been achieved based on these findings.

The present invention provides a heparin-binding protein functionalized by covalently bonding thereto a sugar chain. The sugar chain may be selected from the group consisting of a sulfated polysaccharide, a glycosaminoglycan, an N-linked sugar chain, an O-linked sugar chain and a combination thereof. The heparin-binding protein may be a factor belonging to the FGF family or its allied factor. The heparin-binding protein may be covalently bonded to the sugar chain through a peptide to which the sugar chain can be added. For example, the heparin-binding protein to which the sugar chain is to be covalently bonded may be the following (a) or (b):

(a) a protein consisting of the amino acid sequence of SEQ ID NO: 1, 3, 5, 17, 19, 21, 23, 25, 27 or 29;

(b) a protein which consists of the amino acid sequence of SEQ ID NO: 1, 3, 5, 17, 19, 21, 23, 25, 27 or 29 having deletion, substitution, addition or modification of one or several amino acids, which has FGF activity and to which the sugar chain can be added.

In the heparin-binding protein of the invention, the sugar chain may be bonded to the heparin-binding protein at a site forming a turn in the secondary structure or a site near one of the ends, or a site

which would not change the tertiary structure of the protein greatly by addition of the sugar chain.

The present invention also provides a method for producing a heparin-binding protein functionalized by covalently bonding thereto a sugar chain, comprising the following steps:

(a) a step in which a cDNA coding for a peptide to which a sugar chain can be added is ligated to a cDNA coding for a heparin-binding protein;

(b) a step of incorporating the resultant ligated cDNA into an expression vector;

(c) a step of introducing the expression vector into a host cell having a sugar chain addition pathway; and

(d) a step of expressing in the host cell a heparin-binding protein to which a sugar chain is covalently bonded through the peptide to which the sugar chain can be added.

When the sugar chain is a sulfated polysaccharide or a glycosaminoglycan, the peptide to which the sugar chain can be added may be a proteoglycan core protein or a part thereof. When the sugar chain is an N-linked sugar chain, the peptide to which the sugar chain can be added may be a peptide comprising an N-linked sugar chain-added amino acid sequence. When the sugar chain is an O-linked sugar chain, the peptide to which the sugar chain can be added may be a peptide comprising an O-linked sugar chain-added amino acid sequence. The present invention also provides a method for producing a heparin-binding protein functionalized by covalently bonding thereto a sugar chain, comprising a step of allowing the sugar chain to bind to the heparin-binding protein by a chemical binding method. The sugar chain may be selected from the group consisting of a sulfated polysaccharide, a glycosaminoglycan, an N-linked sugar chain, an O-

linked sugar chain and a combination thereof, and the heparin-binding protein may be a factor belonging to the FGF family or its allied factor. The present invention further provides a pharmaceutical composition containing, as an active ingredient, a heparin-binding protein functionalized by covalently bonding thereto a sugar chain. The present invention also provides a method for functionalizing a natural protein having no sugar chain by covalently bonding thereto a sugar chain.

The novel sugar chain-added heparin-binding protein of the invention is excellent in stabilities such as thermostability, acid resistance, alkali resistance and resistance to proteolytic enzymes. Thus, by using the sugar chain-added heparin-binding protein of the invention in a pharmaceutical product, it is possible to design such a pharmaceutical product that is excellent in *in vivo* stabilities, in particular acid resistance and alkali resistance, and applicable to an oral medicine.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows typical examples of sulfated polysaccharide and glycosaminoglycan sugar chains.

Fig. 2 shows typical examples of N-linked sugar chains.

Fig. 3 shows typical examples of O-linked sugar chains.

Fig. 4 shows SDS-denatured electrophoregrams of various S/FGF-1a-like proteins.

Fig. 5 shows the DNA synthesis promoting activity on HUVEC of S/FGF-1a-II and *E. coli*-derived FGF-1a.

Fig. 6 shows the thermostability, acid resistance and alkali resistance of S/FGF-1a-II and *E. coli*-derived FGF-1a.

Fig. 7 shows the resistance to trypsin of S/FGF-1a-II and *E. coli*-derived FGF-1a.

Fig. 8 shows the DNA synthesis promoting activity on HUVEC of N-FGF-6/1a-IV and *E. coli*-derived FGF-1a.

Fig. 9 shows the heparin affinity of S/FGF-1a-II.

## DESCRIPTION OF PREFERRED EMBODIMENTS

Hereinbelow, the present invention will be described in detail.

In the present invention, the heparin-binding protein to which a sugar chain is to be covalently bonded is a protein having heparin binding property. For example, factors belonging to the FGF family or allied factors, or other proteins with heparin-binding property but without structural similarity to the former proteins may be enumerated. Examples of the other proteins include, but are not limited to, heparin-binding epidermal growth factor-like factor (HB-EGF) and platelet-derived growth factor (PDGF). As specific examples of the factors belonging to the FGF family or allied factors, FGF-1 to -10 and FHF (fibroblast growth factor homologous factor)-1 to -4 are known. The heparin-binding protein of the invention may be covalently bonded to a sugar chain through a peptide to which the sugar chain can be added. For example, the heparin-binding protein to which the sugar chain is to be covalently bonded may be the following (a) or (b):

(a) a protein consisting of the amino acid sequence of SEQ ID NO: 1, 3, 5, 17, 19, 21, 23, 25, 27 or 29;

(b) a protein which consists of the amino acid sequence of SEQ ID NO: 1, 3, 5, 17, 19, 21, 23, 25, 27 or 29 having deletion, substitution, addition or modification of one or several amino

acids, which has FGF activity and to which the sugar chain can be added.

Proteins having the amino acid sequences of SEQ ID NOS: 1, 3, 5, 17, 19, 21, 23, 25, 27 and 29 are encoded by, for example, the DNA sequences of SEQ ID NOS: 2, 4, 6, 18, 20, 22, 24, 26, 28 and 30, respectively. These proteins contain a peptide sequence to which a sugar chain can be added and a sequence for a signal peptide in addition to a peptide sequence for a factor belonging to the FGF family. The heparin-binding protein of the present invention includes not only the protein primarily defined by a cDNA shown in the sequence listing but also a protein in which a peptide sequence for secretion (called the signal peptide) located at the amino terminal when secreted from cells is cut off. The utility of a heparin-binding protein which is contained in the pharmaceutical composition of the invention as an active ingredient will not vary even if the protein is produced in a form lacking the signal peptide from the beginning.

The sugar chain to be covalently bonded to the heparin-binding protein may be any sugar chain as long as the protein is functionalized by covalently bonding the sugar chain. Examples of the sugar chain include, but are not limited to, sulfated polysaccharides such as heparan sulfate, chondroitin sulfate, glycosaminoglycans, N-linked sugar chains and O-linked sugar chains. The term "functionalize" used herein means increasing the activity of a protein of interest. As an example of functionalization, there may be given a case in which the residual activity of a protein after treatment with heat, acid or alkali is increased by adding a sugar chain to the protein by a covalent bond. The "sulfated polysaccharide" used herein is a general term for various sugar chain

structures which are elongating from xylose linked to a serine residue present in the primary structure of proteins or elongating on the non-reducing end side of N-linked sugar chains or O-linked sugar chains to be described later, or which are present in a free form. Many of such sugar chains are composed of repeating disaccharides of aminosugar and uronic acid (or galactose), and some of their hydroxyl groups or amino groups are substituted with sulfate groups. Glycosaminoglycans are polysaccharides having a structure similar to those described above, but they include those which do not have any substitution with sulfate groups. All of the above-mentioned polysaccharides are designated herein generically "sulfated polysaccharides or the like". Their specific structures are described, for example, in Destiny of Sugar Chains in Cells, Nagai, Hakomori and Kobata (Eds.), Kodansha Scientific Co. Fig. 1 shows their typical sugar chain sequences. The "N-linked sugar chain" used herein is a general term for various sugar chain structures elongating from N-acetylglucosamine linked to an asparagine residue present in the primary structure of proteins. Their specific structures are described, for example, in Destiny of Sugar Chains in Cells, Nagai, Hakomori and Kobata (Eds.), Kodansha Scientific Co. Fig. 2 shows their typical sugar chain sequences. The "O-linked sugar chain" used herein is a general term for various sugar chain structures elongating from N-acetylgalactosamine linked to a serine or threonine residue present in the primary structure of proteins. Their specific structures are described, for example, in Destiny of Sugar Chains in Cells, Nagai, Hakomori and Kobata (Eds.), Kodansha Scientific Co. Fig. 3 shows their typical sugar chain sequences. These sulfated polysaccharides or the like, N-linked sugar chains and O-linked sugar chains may have addition, deletion, substitution or modification in a

part of their sugar chain sequences as long as they retain their functions.

When a sugar chain is attached to a heparin-binding protein, the sugar chain alone may be covalently bonded to the heparin-binding protein directly. Alternatively, a peptide chain of any length to which a sugar chain is covalently bonding may be covalently bonded to a heparin-binding protein.

In order to produce the heparin-binding protein of the invention to which a sugar chain is covalently bonded (hereinafter, referred to as the "sugar chain-added heparin-binding protein"), first, a cDNA coding for a peptide to which a sugar chain can be added is ligated to a cDNA coding for a heparin-binding protein. The ligated cDNA is incorporated into an appropriate expression vector, which is then introduced into a host cell having a sugar chain addition pathway to thereby express a sugar chain-added heparin-binding protein.

cDNAs coding for various heparin-binding proteins can be obtained by designing appropriate primers from a sequence registered in a gene bank such as DDBJ (DNA Data Bank of Japan) and performing RT-PCR (reverse transcription PCR) with the primers and mRNA from the relevant tissue of the relevant animal.

In order to produce a sulfated polysaccharide or the like-added heparin-binding protein, first, a cDNA coding for a heparin-binding protein is ligated to a cDNA coding for a peptide which is known to undergo addition of a sulfated polysaccharide or the like. The ligated cDNA is incorporated into an appropriate host cell expression vector, which is then introduced into a host cell to thereby express the sulfated polysaccharide or the like-added heparin-binding protein. As the peptide which is known to undergo addition of a sulfated polysaccharide or the like, the core protein or a part thereof of

various proteoglycans (e.g. syndecan, glypican, perlecan) may be used. As a part of the core protein of a proteoglycan, a peptide comprising a Ser-Gly repeat sequence (which is believed to be the sugar chain addition site in proteoglycans) may be used.

In order to produce an N-linked sugar chain-added heparin-binding protein, first, a cDNA coding for a heparin-binding protein is ligated to a cDNA coding for a peptide which is known to undergo addition of an N-linked sugar chain. The ligated cDNA is incorporated into an appropriate host cell expression vector, which is then introduced into a host cell to thereby express the N-linked sugar chain-added heparin-binding protein. Specific examples of the peptide which is known to undergo addition of an N-linked sugar chain include Asn-X-Thr and Asn-X-Ser (wherein X is any amino acid except proline).

In order to produce an O-linked sugar chain-added heparin-binding protein, first, a cDNA coding for a heparin-binding protein is ligated to a cDNA coding for a peptide which is known to undergo addition of an O-linked sugar chain. The ligated cDNA is incorporated into an appropriate host cell expression vector, which is then introduced into a host cell to thereby express the O-linked sugar chain-added heparin-binding protein. As a specific examples of the peptide which is known to undergo addition of an O-linked sugar chain, Ala-Thr-Pro-Ala-Pro-may be given.

As the site to which a sugar chain is bonded, a site forming a turn in the secondary structure of a heparin-binding protein or a site near one of the ends, or a site which would not change the tertiary structure of the protein greatly by addition of the sugar chain is preferable.

One example of the method for producing a sugar chain-added

heparin-binding protein of the invention will be described below.

First, an oligonucleotide coding for a secretion signal and a peptide which is known to undergo addition of a sugar chain is synthesized or amplified by PCR. The resultant oligonucleotide is incorporated at the 5' end of a plasmid coding for a heparin-binding protein.

As the secretion signal and the peptide which is known to undergo addition of a sugar chain, an amino terminal of a typical secretion-type glycoprotein may be used, for example. Specifically, the amino acid consisting of the N terminal 40 residues of mouse FGF-6 may be used.

The plasmid coding for a heparin-binding protein can be prepared by incorporating a DNA coding for the heparin-binding protein into an appropriate plasmid. As the plasmid into which a DNA coding for a heparin-binding protein is to be incorporated, any plasmid may be used as long as it is replicated and maintained in a host. For example, pBR322 and pUC18 from *E. coli* and pET-3c which was constructed based on these plasmids may be enumerated.

As a method for incorporating the above-described oligonucleotide into the plasmid coding for a heparin-binding protein, the method described in T. Maniatis *et al.*, Molecular Cloning, Cold Spring Harbor Laboratory, p. 239 (1982) may be given, for example.

From the thus prepared plasmid, a region comprising a nucleotide sequence coding for a secretion signal, a peptide which is known to undergo addition of a sugar chain and a heparin-binding protein (hereinafter, referred to as a "region comprising a nucleotide sequence coding for a sugar chain-added heparin-binding protein") is cut out. This region is ligated to the downstream of a promoter in a vector suitable for expression to thereby obtain an expression vector.

The above-described region comprising a nucleotide sequence coding for a sugar chain-added heparin-binding protein may have ATG at its 5' end as a translation initiation codon and TAA, TGA or TAG at its 3' end as a translation termination codon. In order to express the protein encoded in the coding region, a promoter is ligated to the upstream of the region. As the promoter to be used in the present invention, any promoter may be used as long as it is appropriate to the host used for the expression of the gene. When the host to be transformed is a bacillus, SP01 promoter, SP02 promoter, penP promoter or the like may be used. When the host is a yeast, PH05 promoter, PGK promoter, GAP promoter, ADH promoter or the like may be used. When the host is an animal cell, a promoter from SV40 or a promoter from a retrovirus may be used.

As the plasmid into which the thus constructed recombinant DNA comprising a nucleotide sequence coding for a sugar chain-added heparin-binding protein is to be incorporated, any plasmid may be used as long as it can be expressed in the host cell. For example, those vectors which were constructed based on *E. coli*-derived pBR322 and pUC18 may be given.

As a method for incorporating the recombinant DNA into a plasmid, the method described in T. Maniatis *et al.*, Molecular Cloning, Cold Spring Harbor Laboratory, p. 239 (1982) may be given, for example.

By introducing a vector comprising the above-described recombinant DNA into a host cell, a transformant carrying the vector is prepared.

As the host cell, any cell may be used as long as it has a sugar chain addition pathway. Specific examples include, but are not limited to, bacilli (e.g. *Bacillus subtilis* DB105), yeasts (e.g. *Pichia pastoris, Saccharomyces cerevisiae*), animal cells (e.g. COS

cell, CHO cell, BHK cell, NIH3T3 cell, BALB/c3T3 cell, HUVE cell, LEII cell) and insect cells (e.g. Sf-9 cell, Tn cell).

The above-mentioned transformation may be performed by a conventional method commonly used for each host. Alternatively, an applicable method may be used though it is not commonly used. For example, when the host is a yeast, a vector comprising the recombinant DNA is introduced into competent cells (prepared by the lithium method or the like) by the temperature shock method or electroporation. When the host is an animal cell, a vector comprising the recombinant DNA is introduced into cells at the logarithmic growth phase or the like by the calcium phosphate method, lipofection or electroporation.

By culturing the thus obtained transformant in a medium, a sugar chain-added heparin-binding protein is produced. As the medium for culturing the transformant, a conventional medium commonly used for each host may be used. Alternatively, an applicable medium may be used even if it is not commonly used. For example, when the host is a yeast, YPD medium or the like may be used. When the host is an animal cell, Dulbecco's MEM supplemented with animal serum, or the like may be used. The cultivation may be performed under conditions commonly employed for each host. Alternatively, applicable conditions may be used even if they are not commonly used. For example, when the host is a yeast, the cultivation is carried out at about 25-37℃ for about 12 hours to 2 weeks. If necessary, aeration or agitation may be carried out. When the host is an animal cell, the cultivation is carried out at about 32-37℃ under 5% $CO_2$ and 100% humidity for about 24 hours to 2 weeks. If necessary, the conditions of the gas phase may be changed or agitation may be carried out.

In order to obtain a sugar-chain added heparin-binding protein

from the culture of the above-described transformant, the protein released into the culture fluid may be directly recovered from a supernatant after centrifugation. Alternatively, when the protein is to be extracted from the cultured microorganisms or cells, the protein may be obtained by disrupting the cultured microorganisms or cells with a homogenizer, a French press, ultrasonic waves, lysozyme and/or by freeze-thawing to thereby elute the protein of interest to the outside of the cells, and then recovering the protein from soluble fractions. If the protein of interest is contained in insoluble fractions, insoluble fractions may be recovered by centrifugation after disruption of the microorganisms or cells and then solubilized with a buffer containing guanidine hydrochloride or the like, to thereby recover the protein of interest from the resultant soluble fractions. Alternatively, the cultured microorganisms or cells may be disrupted by a direct treatment with a buffer containing a protein denaturing agent such as guanidine hydrochloride to thereby elute the protein of interest to the outside of the cells.

In order to purify a sugar chain-added heparin-binding protein from the above-mentioned supernatant, known separation/purification methods may be used in an appropriate combination. Specific examples of these known separation/purification methods include salting out, solvent precipitation, dialysis, ultrafiltration, gel filtration, SDS-polyacrylamide gel electrophoresis, ion exchange chromatography, affinity chromatography, reversed phase high performance liquid chromatography and isoelectric focusing. Further, affinity chromatography using heparin sepharose as a carrier may be applicable to a large number of heparin-binding proteins.

The thus obtained sample may be dialyzed and freeze-dried to

obtain dry powder if the activity of the sugar chain-added heparin-binding protein is not damaged by such processing. Further, in storing the sample, addition of serum albumin to the sample is effective for preventing adsorption of the sample to the container.

The inclusion of an extremely small amount of a reducing agent in the purification process or the storing process is preferable for preventing oxidation of the sample. As the reducing agent, β-mercaptoethanol, dithiothreitol, glutathione or the like may be used.

The sugar chain-added heparin-binding protein of the invention may also be produced by attaching a sugar chain to a heparin-binding protein by a chemical method. As the specific method, the following a) or b), or a combination thereof may be used.

a) For example, first, a sugar chain is completed by a biological method, a chemical synthesis method or a combination thereof. At that time, a residue appropriate for protein binding may be introduced at one end of the sugar chain. For example, an aldehyde group is formed by reducing and partially oxidizing the reducing end of the completed sugar chain. Then, this aldehyde group is attached to an amino group in a protein by an amino bond to thereby complete the joining of the sugar chain and the protein.

b) For example, first, an aldehyde group is formed by reducing and partially oxidizing the reducing end of a monosaccharide or a residue appropriate for protein binding which is bound to a monosaccharide. Then, this aldehyde group is attached to an amino group in a protein by an amino bond to thereby complete the joining of the monosaccharide and the protein. An additional monosaccharide or sugar chain is attached to a hydroxyl group or the like of the above monosaccharide to thereby complete a sugar chain. For this attachment, a biological method, a chemical synthesis method or a

combination thereof may be considered.

A heparin-binding protein functionalized by covalently bonding thereto a sugar chain can be used as a medicine. For example, the sugar chain-added heparin-binding protein of the invention regulates the physiological function of FGF. Specifically, the physiological function of FGF is to promote or inhibit the growth of fibroblast, vascular endothelial cell, myoblast, cartilage cell, osteoblast and glia cell. Therefore, the sugar chain-added heparin-binding protein of the invention is effective for promoting cell growth and tissue regeneration in liver or the like; for curing wounds and regulating nervous function; and for regulating the growth of fibroblast or the like. The protein of the invention is useful for preventing or treating various diseases such as fibroblastoma, angioma, osteoblastoma, death of neurocytes, Alzheimer's disease, Parkinson's disease, neuroblastoma, amnesia, demensia and myocardial infarction. The protein of the invention can also be used as a trichogenous agent or a hair-growing agent.

The sugar chain-added heparin-binding protein obtained as described above may be formulated into pharmaceutical compositions such as liquid, lotions, aerosols, injections, powder, granules, tablets, suppositories, enteric coated tablets and capsule, by mixing the protein with pharmaceutically acceptable solvents, vehicles, carriers, adjuvants, etc. according to conventional formulation methods.

The content of the sugar chain-added heparin-binding protein, which is an active ingredient, in the pharmaceutical composition may be about 0.0000000001 to 1.0% by weight.

The pharmaceutical composition can be administered parenterally or orally to mammals, e.g. human, mouse, rat, rabbit, dog, cat, etc.

in a safe manner. The dose of the pharmaceutical composition may be appropriately changed depending on the dosage form, administration route, conditions of the patient and the like. For example, for administration to mammals including human, 0.0001 to 100 mg of the sugar chain-added heparin-binding protein may be applied to the diseased part several times a day.

The present invention has been described so far taking heparin-binding proteins as an example. However, it should be noted that besides the heparin-binding proteins, natural proteins having no sugar chain can also be functionalized by covalently bonding thereto a sugar chain.

## DEPOSIT OF MICROORGANISMS

Clones of *E. coli* DH5$\alpha$ carrying plasmids incorporating genes coding for the sugar chain-added heparin-binding proteins of the invention (having the DNA sequences of SEQ ID NOS: 2, 4, 18, 20, 22, 24, 26, 28 and 30, respectively) were deposited at the National Institute of Bioscience and Human-technology, Agency of Industrial Science and Technology under Accession Numbers of FERM BP-6428, FERM BP-6424, FERM BP-6427, FERM BP-6431, FERM BP-6429, FERM BP-6430, FERM BP-6423, FERM BP-1625 and FERM BP-6426 on September 10, 1997.

Hereinbelow, the present invention will be described specifically with reference to the following Example. However, the present invention is not limited to this Examples.

[Example 1]

1) Construction of S/FGF-1a-II Plasmid

1. Preparation of a Human Ryudocan cDNA Fragment

phR7A8 is a plasmid obtained by inserting a human ryudocan cDNA (PCR product) into the EcoR V site of pBluescript II (KS+) cloning vector. This plasmid contains a partial sequence from position 7 to position 2610 in the mRNA sequence shown under Accession No. D13292 (see B.B.R.C. Vol. 190, No. 3, pp. 814-822, 1993).

This plasmid was digested with Pvu II. Using the resultant DNA fragment of 2,232 base pairs as a template, a PCR (polymerase chain reaction) was performed. As primers, #109 (5'-TTG TCG ACC CAC CAT GGC CCC CGC CCG TCT-3') (SEQ ID NO: 7) and #111 (5'-TTG ATA TCT AGA GGC ACC AAG GGA TG-3')(SEQ ID NO: 8) were used. The specifically amplified 276 bp band was separated by electrophoresis. After extraction, this fragment was double-digested with EcoR V and Sal I. The resultant 268 bp band was separated, extracted and then used in the ligation described below.

2. FGF-1a/pBluescript II (KS+)

A PCR was performed using human FGF-1 cDNA as a template and #967 (5'-GCG TCG ACA GCG CTA ATT ACA AGA AGC CCA AAC TC-3')(SEQ ID NO: 9) and #630 (5'-CCG AAT TCG AAT TCT TTA ATC AGA AGA GAC TGG-3') (SEQ ID NO: 10) as primers. The specifically amplified 434 bp band was separated by electrophoresis. After extraction, this fragment was double-digested with EcoR I and Sal I. The resultant 422 bp band was separated, extracted and then inserted into pBluescript II (KS+) cloning vector (2934 bp) double-digested with EcoR I and Sal I, where upon FGF-1a/pBluescript II (KS+) was produced.

FGF-1a/pBluescript II (KS+) was digested with Aor51H I and Sal I in this order. The resultant 2626 bp band was separated, extracted and then used in the ligation described below.

3. Preparation of S/FGF-1a-II Chimeric Gene

EcoR V/Sal I fragment (a PCR product from human ryudocan) and Aor51H I/Sal I fragment from FGF-1a/pBluescript II (KS+) were subjected to a DNA ligation to produce S/FGF-1a-II/pBluescript II (KS+) vector. Subsequently, this vector was double-digested with EcoR I and Sal I to give a 678 bp band, which was then separated and extracted. The resultant fragment was inserted into pMEXneo expression vector (5916 bp) double-digested with EcoR I and Sal I, where upon S/FGF-1a-II/pMEXneo was produced. This expression vector comprises the nucleotide sequence shown in SEQ ID NO: 2.

2) Expression of S/FGF-1a-II

The resultant S/FGF-1a-II/pMEXneo was transferred into CHO-K1 cells (Chinese hamster ovary cell K1 substrain) by lipofection. Then, the cells were cultured in the presence of Geneticin to select gene-transferred cells. The selected cells were grown until the culture plate became almost full. Then, the medium was exchanged with a serum-free medium to increase the substance productivity of the cells. Thereafter, the medium was exchanged with a fresh one every two days. The resultant conditioned medium was subjected to low speed centrifugation, and the resultant supernatant was stored at 4℃.

3) Construction of N-FGF-6/1a-IV Plasmid

1. Preparation of a Mouse FGF-6 cDNA Fragment

A PCR was performed using mouse FGF-6 cDNA as a template and #1048 (5'-GCG TCG ACC CAC CAT GTC CCG GGG AGC AGG ACG TGT TCA GGG CAC GCTGCA GGC TCT CGT CTT C-3')(SEQ ID NO: 11) and #968 (5'-GCG ATA TCC AGT AGC GTG CCG TTG GCG CG-3')(SEQ ID NO: 12) as primers. The specifically amplified 138 bp band was separated by electrophoresis.

After extraction, this fragment was double-digested with EcoR V and Sal I. The resultant 130 bp band was separated, extracted and then used in the ligation described below.

2. Preparation of N-FGF-6/1a-IV Chimeric Gene

EcoR V/Sal I fragment (a PCR product from mouse FGF-6) and Aor51H I/Sal I fragment from FGF-1a/pBluescript II (KS+) were subjected to a DNA ligation to produce N-FGF-6/1a-IV/pBluescript II (KS+) vector. Subsequently, this vector was double-digested with EcoR I and Sal I to give a 540 bp band, which was then separated and extracted. The resultant fragment was inserted into pMEXneo expression vector (5916 bp) double-digested with EcoR I and Sal I, where upon N-FGF-6/1a-IV/pMEXneo was produced. This expression vector comprises the nucleotide sequence shown in SEQ ID NO: 4.

4) Expression of N-FGF-6/1a-IV

N-FGF-6/1a-IV was secreted into a culture supernatant by transferring N-FGF-6/1a-IV/pMEXneo into CHO-K1 cells in the same manner as described above for S/FGF-6/1a-II.

5) Construction of O-FGF-6/1a Plasmid

1. Preparation of N-FGF-6/1a<NQ> Chimeric Gene

A PCR was performed using N-FGF-6/1a/pBluescript II (KS+) vector as a template and #105 (5'-GCG TCG ACC CAC CAT GTC-3')(SEQ ID NO: 13) and #124 (5'-GCG ATA TCC AGT AGC GTG CCT TGG GCG CG-3')(SEQ ID NO: 14) as primers. The specifically amplified 138 bp band was separated by electrophoresis. After extraction, this fragment was double-digested with EcoR V and Sal I. The resultant 130 bp band was subjected to the ligation described below together with Aor51H I/Sal

I fragment from FGF-1a/pBluescript II (KS+), to thereby yield N-FGF-6/1a<NQ>/pBluescript II (KS+) vector.

2. Preparation of O-FGF-6/1a Chimeric Gene

A primary PCR was performed using N-FGF-6/1a<NQ>/pBluescript II (KS+) vector as a template and #098 (5'-GCT GGA GGA GGC TGC TAC TCC AGC TCC AAA CCA TTA CA-3')(SEQ ID NO: 15) and #116 (5'-GCC GCT CTA GAA CTA GTG GAT-3')(SEQ ID NO: 16) as primers. The specifically amplified 210 bp band was purified. Using this PCR product and #115 (5'-AAC AAA AGC TGG GTA CCG GG-3') as primers, a secondary PCR was performed. The specifically amplified 631 bp band was separated by electrophoresis. After extraction and purification, this fragment was double-digested with EcoR I and Sal I. The resultant 558 bp band was separated, extracted and then inserted into pMEXneo expression vector (5916 bp) double-digested with EcoR I and Sal I, to thereby yield O-FGF-6/1a/pMEXneo. This expression vector comprises the nucleotide sequence shown in SEQ ID NO: 6.

6) Expression of O-FGF-6/1a

O-FGF-6/1a was secreted into a culture supernatant by transferring O-FGF-6/1a/pMEXneo into CHO-K1 cells in the same manner as described above for S/FGF-1a-II.

7) Expression of FGF-1a in *E. coli*

The fragment from human FGF-1a cDNA obtained by double digestion with Eco RI and Sal I as described above was incorporated into an *E. coli* expression vector pET3c. *E. coli* BL21(DE3)pLysS was transformed with the resultant vector. Subsequently, the transformant at the logarithmic growth phase was stimulated with IPTG (isopropylthio- $\beta$

-galactoside) to induce the expression of the transferred gene. The cells were collected and sonicated for disruption to thereby release FGF-1a, which was then recovered in a centrifugation supernatant.

8) Removal of N-Linked Sugar Chains by Peptide N-Glycosidase F Treatment

N-FGF-6/1a-II concentrated with heparin-Sepharose beads was boiled and eluted in an electrophoresis buffer, as will be described later (see Test Example 1). To a part of the resultant solution, NP-40 (final concentration: 1%), Tris-HCl buffer (pH 7.5) and peptide N-glycosidase F (0.3 U) were added and the mixture was kept at 37℃ overnight. Then, the solution was heated at 100℃ for 3 min to terminate the enzyme reaction. This reaction solution was analyzed by SDS-denatured electrophoresis, as will be described later.

Various S/FGF-1a and N-FGF-6/1a genes can be prepared by appropriately altering the PCR primers (#111 and #968) used in "1. Preparation of a Human Ryudocan cDNA Fragment" and "1. Preparation of a Mouse FGF-6 cDNA Fragment" in the above Example and by replacing the restriction enzyme EcoR V with an appropriate enzyme which would generate a blunt end. Examples of such cDNA sequences are shown in SEQ ID NOS: 8, 20, 22, 24, 26 and 28.

Various O-FGF-6/1a genes can be prepared by replacing the template used in the PCR in "2. Preparation of O-FGF-6/1a Chimeric Gene" above with S/FGF-1a-II/pBluescript II (KS+), N-FGF-6/1a-IV/pBluescript II (KS+) or the like, or by appropriately altering the PCR primers (#098, #116 and #115), or by a combination of the both methods. An example of such a cDNA sequence is shown in SEQ ID NO: 30.

[Test Example 1] SDS-Denatured Electrophoresis

Heparin Sepharose beads added to conditioned media of various FGF-1a-like proteins-secreting cells were individually washed and then boiled directly with an electrophoresis buffer (containing SDS and 2-mercaptoethanol). The eluted protein was used as a sample. This sample was electrophoresed on 12.5% acrylamide gel in the presence of SDS and 2-mercaptoethanol. After being electrically transferred onto a nitrocellulose membrane, the protein was stained with anti-FGF-1 monoclonal antibody and horseradish peroxidase-labelled anti-mouse IgG antibody, followed by detection by the chemiluminescence method (Fig. 4). In the Figure, the arrows at the left side indicate the locations of standard proteins with known molecular weights and their molecular weights (in daltons). Panel A) shows an SDS-denatured electrophoregram of S/FGF-1a-II. Panel B) shows SDS-denatured electrophoregrams of FGF-1a produced in *E. coli* (lane a); N-FGF-1a-IV obtained by treating N-FGF-6/1a-IV with peptide N-glycosidase F for removal of N-linked sugar chains (lane b); N-FGF-6/1a-IV (lane c) and O-FGF-6/1a (lane d).

[Test Example 2] DNA Synthesis Promoting Activity

The cell cycle of HUVEC (human umbilical cord-derived vascular endothelial cell) stops even in the presence of 15% serum if growth factors such as FGF are lacking. S/FGF-1a-II, N-FGF-6/1a-IV, O-FGF-6/1a, or FGF-1a produced in *E. coli* was added to HUVEC in such a state. Eighteen hours later, radio-labelled thymidine was allowed to be taken up for 6 hours. The amount of radioactivity taken up into DNA during this period was regarded as indicating the amount of the newly synthesized DNA.

1. DNA Synthesis Promoting Effect (Heparin Non-Dependent) of S/FGF-1a-II on Human Vascular Endothelial Cell

A conditioned medium was prepared from a serum-free medium of S/FGF-1a-II gene-transferred cells. This conditioned medium was dialyzed against PBS and then added to HUVEC in the presence (5 $\mu$ g/ml) or absence of heparin, for examining the DNA synthesis promoting activity of S/FGF-1a-II on HUVEC. As a result, unlike FGF-1a produced in *E. coli*, S/FGF-1a-II promoted the DNA synthesis of HUVEC in a non-heparin-dependent manner (Fig. 5).

2. DNA Synthesis Promoting Effect of N-FGF-6/1a-IV on Human Vascular Endothelial Cell

A conditioned medium was prepared from a serum-free medium of N-FGF-6/1a-IV gene-transferred cells. This conditioned medium was dialyzed against PBS and then added to HUVEC in the presence (5 $\mu$ g/ml) or absence of heparin, for examining the DNA synthesis promoting activity of N-FGF-6/1a-IV on HUVEC. As a result, like FGF-1a produced in *E. coli*, N-FGF-6/1a-IV promoted the DNA synthesis of HUVEC. However, its heparin dependency was weak, and N-FGF-6/1a-IV exhibited stronger DNA synthesis promoting activity than FGF-1a from *E. coli* in the absence of heparin (Fig. 8).

[Test Example 3] Heparin Affinity Chromatography

The heparin affinity of S/FGF-1a-II obtained in 2) in the above Example was examined. Heparin-Sepharose beads were added to a conditioned medium of S/FGF-1a-II-secreting cells and agitated at 4 ℃ for 2 hours or more. Beads precipitating by low speed centrifugation were recovered and washed sufficiently in physiological PBS

(phosphate buffered saline, pH 7.4), followed by elution of the protein bound to heparin-fixed beads with PBS containing 2.5 M NaCl. After addition of distilled water to lower the salt concentration, this eluate was again applied to a high performance liquid chromatography column packed with heparin affinity beads. S/FGF-1a-II was eluted using NaCl density gradient.

While FGF-1a from *E. coli* was eluted at about 1.0 M NaCl, S/FGF-1a-II was eluted at about 0.4 M NaCl. Thus, it appears that affinity to the fixed heparin is lowered in S/FGF-1a-II (Fig. 9). The small peak seen around 1.0 M NaCl in Fig. 9 is considered to be a degradation product from S/FGF-1a-II as analyzed by SDS-denatured electrophoresis.

[Test Example 4] Thermostability of FGF-1a-Like Proteins

Conditioned media of various FGF-1a-like protein-secreting cells were individually dialyzed against PBS sufficiently. A part of each of the resultant media was retained in PBS kept at 56℃ or 70℃ for 30 minutes, or retained at room temperature for 12 hours. Thereafter, the medium was re-dialyzed against PBS at 4 ℃ to prepare a sample. The stability of S/FGF-1a-II was determined by subjecting it to DNA synthesis promoting activity test on HUVEC after various treatments and then comparing the resultant activity with the activity of an S/FGF-1a-II sample dialyzed against PBS at 4 ℃ for 12 hours.

After retention at room temperature for 12 hours, even the activity of *E. coli*-derived FGF-1a was protected by heparin, but the activity of S/FGF-1a-II was protected regardless of the presence or absence of heparin.

After heat treatment at 56 ℃ for 30 minutes, *E. coli*-derived FGF-1a was almost deactivated, but S/FGF-1a-II retained about 50% of

the activity. Thus, it was considered that its thermostability was improved (Fig. 6).

[Test Example 5] Acid Resistance and Alkali Resistance of FGF-1a-Like Proteins

Conditioned media of various FGF-1a-like protein-secreting cells were individually dialyzed against PBS sufficiently. A part of each of the resultant media was dialyzed in a citrate buffer (pH 4.0) or a sodium carbonate buffer (pH 10.0) for 12 hours and then re-dialyzed against PBS at 4 ℃ to prepare a sample. The stability of S/FGF-1a-II was determined by subjecting it to DNA synthesis promoting activity test on HUVEC after various treatments and then comparing the resultant activity with the activity of an S/FGF-1a-II sample dialyzed against PBS at 4 ℃ for 12 hours.

The activity of S/FGF-1a-II decreased little even after acid treatment at pH 4.0 regardless of the presence or absence of heparin; thus, an improvement in acid resistance was recognized (Fig. 6). After alkali treatment at pH 10.0, *E. coli*-derived FGF-1a was almost deactivated, but S/FGF-1a-II retained about 50% of the activity; thus, an improvement was also recognized in alkali resistance (Fig. 6).

[Test Example 6] Stability of FGF-1a-Like Proteins against Proteolytic Enzymes

Conditioned media of various FGF-1a-like protein-secreting cells were individually dialyzed against PBS sufficiently. To a part of each of the resultant media, trypsin solutions of varying concentrations (0.0001-0.1%) were added and kept at 37 ℃ for 1 hour. The thus obtained sample was subjected to the SDS-denatured electrophoresis described previously. The intensity of the remaining

band was compared to the intensity of the band generated by the sample before trypsin treatment to give an indicator of stability.

As a result, as shown in Fig. 7, 88% and 35% of the band intensity remained in S/FGF-1a-II after 0.001% and 0.01% trypsin treatment, respectively; however, the band intensity of *E. coli*-derived FGF-1a decreased to 58% and even to 6% after 0.001% and 0.01% trypsin treatment, respectively. Thus, it was considered that the resistance of S/FGF-1a-II to proteolytic enzymes was increased (Fig. 7).